

# Mechanisms of Fiber-induced Genotoxicity

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The mechanisms of particle-induced genotoxicity have been investigated mainly with asbestos fibers. The results are summarized and discussed in this paper. DNA damage can be produced by oxidoreduction processes generated by fibers. The extent of damage yield depends on experimental conditions: if iron is present, either on fibers or in the medium, damage is increased. However, iron reactivity does not explain all the results obtained in cell-free systems, as breakage of plasmid DNA was not directly associated with the amount of iron released by the fibers. The proximity of DNA to the site of generation of reactive oxygen species (ROS) is important because these species have an extremely short half-life. Damage to cellular DNA can be produced by oxidoreduction processes that originate from cells during phagocytosis. Secondary molecules that are more stable than ROS are probably involved in DNA damage. Oxidoreduction reactions originating from cells can induce mutations. Genotoxicity is also demonstrated by chromosomal damage associated with impaired mitosis, as evidenced by chromosome missegregation, spindle changes, alteration of cell cycle progression, formation of aneuploid and polyploid cells, and nuclear disruption. In some of these processes, the particle state and fiber dimensions are considered important parameters in the generation of genotoxic effects. — *Environ Health Perspect* 105(Suppl 5):1073–1084 (1997)

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## Introduction

In the past, asbestos fibers have been considered nongenotoxic carcinogens (1,2) because of their failure to induce gene mutation in most short-term assays (3) and their ability to exert effects similar to those observed with promoters (4,5). More recently, a study of databases on the effects of nongenotoxic carcinogens, including asbestos, has revealed that many of the compounds tested in short-term bioassays induced chromosomal mutations or aneuploidy (6). New assays have been developed to better investigate the effects of asbestos at the genetic level. The data will be reviewed here and results will be discussed in terms of the mechanisms of action of fibers. The background leading to development of these new assays will be summarized first.

Genotoxicity is one of the key events in neoplastic transformation induced by xenobiotics, as genetic alterations are fundamental changes arising in cancer cells. Neoplasia is associated with permanent genetic changes in critical genes, especially protooncogenes and tumor suppressor genes (7–11). Genotoxicity is a rather loose term that in a narrow sense refers to the property of a substance reacting directly or after metabolic activation with DNA, but, in a broad sense, refers to an agent that damages genetic material. Thus, agents that produce gene mutations at specific loci, as well as DNA damage and repair of damage, aneuploidy, and chromosome mutations, can be considered potentially harmful to the genome.

It has been known for many years that genetic changes are of central importance in tumor development (12). Chromosome abnormalities have been noted frequently in tumors. With the development of more sensitive analytical methods, numerical and structural chromosome abnormalities such as deletions and rearrangements (translocations, amplifications, insertions) have been found in tumors, supporting the hypothesis that chromosome defects are fundamental in neoplasia (13,14). Chromosome and gene mutations now appear as obligatory steps in oncogenesis (15). Neoplastic transformation is a multistep process and multiple genetic changes are necessary to achieve transformation. This is illustrated by the multiple gene and chromosome abnormalities noted in certain cancers, such as colon cancer (16). This type of tumor is characterized by deletions of chromosomes 5, 17, and 18, as well as mutations in the genes *ras* and *p53*, and may serve as a paradigm for neoplastic transformation. From the study of human tumors, it appears that nonrandom genetic changes occur in cancer cells. While defects can be found in numerous genes involving a wide range of diseases, only changes occurring in specific genes are significant in neoplasia. These genes are oncogenes and tumor suppressor genes, which are activated and inactivated in tumor cells, respectively (15).

Several conditions affect the rate of gene and chromosome mutations and thus can enhance the risk of neoplastic cell transformation. There are dominant heritable predisposition syndromes resulting from germline mutations (7), genetic instability syndromes (17), defects in breakage and repair processes, and increased proliferation (7,18,19). Genetically altered cells undergo several stages to complete neoplastic transformation, and additional factors are important in tumor expression. DNA damage is generally repaired by different mechanisms depending on the nature of the deleterious agent. Thus, repair processes are important in neoplastic transformation and error-prone mechanisms may facilitate transformation (20). Cell division is another major factor in transformation processes. Controlled cell division is a component of tissue homeostasis and induced mitogenesis can enhance cell division, producing amplification of mutations in genetically altered cells. Mitogenesis can be induced by the release of growth factors resulting from inflammatory processes.

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Abbreviations used: CH, Chinese hamster; CHO, Chinese hamster ovary; DF, desferrioxamine; 8-OHdG, 8-hydroxydeoxyguanosine; *HGPRT*, hypoxanthine guanine phosphoribosyl transferase; HMC, human mesothelial cells;  $H_2O_2$ , hydrogen peroxide; LOH, loss of heterozygosity; MMMF, man-made mineral fiber(s); MMVF, man-made vitreous fiber(s); OH $\cdot$ , hydroxyl radical; RCF, refractory ceramic fiber; ROS, reactive oxygen species; SHE, Syrian hamster embryo.

Finally, among factors controlling tumor formation, immunological surveillance is important in elimination of abnormal cells.

## Methods for Genotoxicity Assessment

### General Methods

Several methods have been developed to assess genotoxicity and test for carcinogenicity of chemicals either *in vitro* or *in vivo* (Table 1). *In vitro* tests include the detection of gene mutations in procaryotes and eucaryotes (21). Other methods are based on cytogenetic analysis for chromosome aberrations during metaphase, in order to determine numerical and structural chromosome abnormalities, and the formation of micronuclei that arise after completion of mitosis (22–28). While most of these assays have been developed to investigate the effects of chemicals, specific assays have been conducted to study the genotoxicity of fibers and particulate materials. It should be emphasized that there are important differences between chemicals and particulates; chemical agents may enter the body via different routes of exposure and can interact directly with DNA or indirectly following metabolism. In contrast, most particles, with the exception of some drugs, enter the body by inhalation. With particulates, no metabolism occurs; however, internalization and phagocytosis must be considered when studying fiber–cell interactions. Therefore, general methods as well as specific systems have been developed to investigate the potential genotoxicity of fibers.

**Table 1.** Test systems for genotoxicity assessment.

| Structure   | End point   |
|-------------|---|
| DNA         | <i>In vitro</i><br>Growth of revertants (bacteria)<br>Gain/loss of viability (mutations at ouabain, <i>HGPRT</i> , <i>TK</i> loci)  |
|             | <i>In vivo</i><br>DNA adducts, DNA repair   |
| Chromosomes | <i>In vitro</i><br>Structural chromosome aberrations (gaps, breaks, fragments, exchanges, translocations)<br>Numerical chromosome changes (aneuploidy, polyploidy)<br>Sister chromatid exchanges<br>Formation of micronucleus (analysis of cells in interphase) |
|             | <i>In vivo</i><br>Chromosome aberrations, micronucleus  |

*TK*, thymidine kinase.

### Methods Used to Investigate the Genotoxicity of Fibers

Table 2 summarizes *in vitro* assays used to investigate genotoxic potential of fibers. A number of *in vitro* systems have been applied using conventional cell models (Chinese hamster ovary [CHO] cells, mouse and hamster embryo fibroblasts, lung fibroblasts, epithelial cells, and pleural mesothelial cells from different species). In contrast, few *in vivo* studies have been conducted.

**Cell-free Systems Investigating DNA Damage.** Recently, many investigators emphasized the potential of fibers to produce active oxygen species, especially radicals (29). These molecules derive from oxygen ( $O_2$ ) dissolved in the incubation media catalyzed by redox sites at the fiber surface, especially in the presence of Fe. Radicals may be produced by the Haber Weiss mechanism in the presence of Fe and provide hydrogen peroxide ( $H_2O_2$ ) that can be transformed via a Fenton reaction *d*. Alternatively, fiber-treated cells can produce superoxide anion  $O_2^{\cdot-}$ :

- $O_2 + Fe(II) \rightarrow O_2^{\cdot-} + Fe(III)$
- $Fe(III) + O_2^{\cdot-} \rightarrow O_2 + Fe(II)$
- $2 O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$
- $Fe(II) + H_2O_2 \rightarrow OH^{\cdot} + OH^- + Fe(III)$
- $H^+ + OH^- \rightarrow H_2O$

Since reactive oxygen species (ROS) are potentially harmful to biomolecules, including DNA (30), cell-free systems have been developed to investigate DNA damage due to fibers. Desferrioxamine (DF) is often used to block the reaction because its chelation of Fe(III) prevents the reaction *b*

**Table 2.** *In vitro* cellular assays performed to investigate the genotoxic potential of fibers and their mechanisms of action.

| Nature of the assay                      | End point   |
|--|---|
| Detection of gene mutations              | Revertants (bacteria)<br>Gain/loss of cell viability [mutations at ouabain, <i>HGPRT</i> , <i>TK</i> loci, and other genes (eucaryotes)]  |
| Tests indicating clastogenicity          | DNA strand breaks (alkaline elution, nick translation, poly(ADP)ribose polymerase activation, DNA unwinding)<br>Structural chromosome abnormalities<br>Formation of micronuclei                   |
| Cytogenetic aberrations                  | Sister chromatid exchanges<br>Chromosome mutations (deletions, translocations, exchanges) detected by banding or Southern blot analysis<br>Aneuploidy, polyploidy<br>Abnormal mitosis (anaphases) |
| Direct or indirect interactions with DNA | Formation of 8-OHdG<br>DNA repair and related protein expression<br>Cell cycle control  |

**Table 3.** Cell-free *in vitro* assays to investigate fiber-induced DNA damage.

| System                               | End point most commonly used   |
|--------------------------------------|--|
| DNA + fibers<br>± EDTA<br>± $H_2O_2$ | Amount of 8-OHdG/ $10^5$ dG  |
| Plasmid + fibers                     | Proportion of supercoiled, relaxed form, linearized DNA (gel electrophoresis)<br>DNA single strand or double-strand breaks |

and availability of Fe(II). Several end points have been used to identify DNA damage: guanine hydroxylation, changes in DNA structure, DNA breakage, and DNA adducts. Table 3 summarizes test systems that have been developed. The principles will be briefly discussed here.

Hydroxyl radical ( $OH^{\cdot}$ ) may produce dehydroxyguanosine hydroxylation at the C8 position of guanine (Figure 1). In the depicted assays, DNA is generally incubated with the fibers in the presence or absence of different compounds.  $H_2O_2$  and EDTA enhance the reaction. The effect of fibers on DNA structure is determined using plasmid DNA where electrophoresis allows the analysis of conformational changes in DNA.

**In Vitro Systems Investigating DNA Damage to Mammalian Cells or Procaryotes (except Mutagenesis).** In these assays, mammalian cells or procaryotes were incubated with fibers for different periods of time and at different fiber concentrations, depending on the experiment (Table 4). Guanine hydroxylation was measured as described with acellular systems. DNA was extracted from fiber-treated cells and 8-hydroxydeoxyguanosine (8-OHdG)

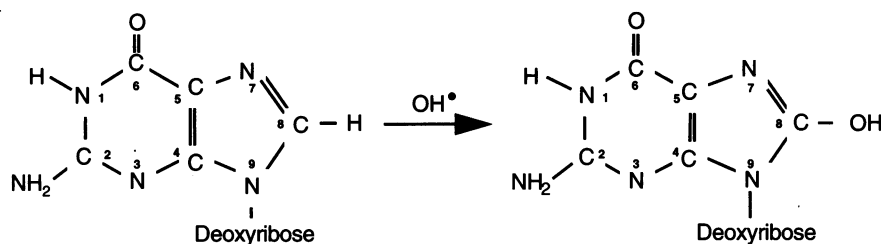


Figure 1. Structure of deoxyguanosine and 8-hydroxydeoxyguanosine.

Table 4. *In vitro* assays developed with cell systems to investigate DNA cell damage after treatment with fibers.

| Procedure   | End point   |
|---|---|
| DNA extraction  | 8-OHdG analysis   |
| DNA analysis by alkaline elution<br>(hydroxyapatite columns, elution through filters) | DNA fluorescence or radioactivity in prelabeled DNA                       |
| Fluorescence analysis of DNA unwinding  | Fluorescence of ethidium bromide  |
| DNA extraction  | Fluorescence due to DNA adducts   |
| Nick translation with labeled nucleotide in the<br>presence of DNA polymerase         | Amount of labeled nucleotide incorporated in the nucleus                  |
| Extraction of nuclear proteins  | Measurement of poly(ADP)ribose polymerase activity                        |
| Addition of labeled nucleotide to growth-<br>arrested cells                           | Amount of labeled nucleotide incorporated in the DNA,<br>i.e., DNA repair |

detected by appropriate methods. DNA strand breaks can also be studied using an alkaline elution procedure with alkaline unwinding and ethidium bromide to detect DNA strand breaks. In addition, the formation of DNA breaks can be determined by indirect methods such as *in situ* labeling of the cells. With this method, if gaps are formed in DNA, they are repaired by incorporation of labeled nucleotide in the presence of DNA polymerase. Other systems have investigated DNA repair, considered evidence of previous DNA damage. Finally, some authors have studied the interaction of lipid-derived molecules with DNA, as fibers produce lipid peroxidation (31,32). Fluorescent products are formed following reaction of malonaldehyde and arachidonic acid metabolites with DNA (33).

**Cellular Systems Investigating a Clastogenic Potency Detected at the Chromosome Level.** *In vitro* cytogenetic assays for chromosome aberrations provide information on clastogenic potency (Table 2). Primary damage to chromosomes consists of breakage of a chromatid; these lesions can be observed in metaphase when exponentially growing cells are incubated in the presence of fibers. Structural chromosome aberrations (breaks, fragments, exchanges of chromosomal segments between two chromosomes) can be observed during the first round of replication. After

completion of cell division, chromosome fragments can be surrounded by a nuclear membrane after completion of mitosis and counted in interphase cells as micronuclei. Whole chromosomes may also be found in micronuclei. These abnormalities reflect chromosome breakage. The type of aberrations depends on the type of damage, position in the cell cycle, and cell repair processes (34). Several types of mammalian cells can be used to investigate chromosomal damage. Chromosome breakage may result in morphological changes in chromosomes. Translocations, inversions, and deletions may be a consequence of rearrangements following breakage. Sister chromatid exchanges can be produced by recombination. Chromosome mutations involving a large segment of the chromosome can be visualized by chromosome banding methods. Smaller deletions, translocations, or inversions can be detected by Southern blot analysis or *in situ* hybridization using specific probes.

**Studies of Aneuploidy and Polyploidy.** The number of chromosomes is maintained by appropriate segregation of chromosomes in daughter cells during mitosis. If the mechanism of chromosome segregation is impaired, the progeny will contain an abnormal number of chromosomes. Nondisjunction is an important genetic risk; this phenomenon will result in an incorrect genetic dosage in the daughter

cells (35,36). Aneuploidy can be determined by counting the number of chromosomes in metaphase spreads. Aneuploidy resulting from fiber exposure has been studied in several types of mammalian cells. Other protocols have been developed to determine the effects of fibers on chromosome segregation, in particular, analysis of anaphase and telophase. Polyploidy may result from endoreplication and/or from an impairment of cytokinesis.

**Gene Mutation Assays.** The most widely used *in vitro* gene mutation assays are based on reverse mutation in *Salmonella typhimurium* (21) or in *Escherichia coli*. These assays have been used to investigate the mutagenic potential of fibers. Mammalian mutation gene protocols have also been used, particularly those involving mutation at the hypoxanthine guanine phosphoribosyl transferase (*HGPRT*) locus, thymidine kinase locus, and  $\text{Na}^+/\text{K}^+$  ATPase. In these assays, mammalian cells are incubated with a precursor that is metabolized to a toxic compound or becomes toxic following interaction with a specific protein. The consequence is a loss of cell viability; retention of viable cells is associated with mutations at a specific gene locus. These protocols are based on the selection of viable cells following mutations under conditions when wild-type cells are killed; therefore, the mutated gene must be nonessential for cell viability. Moreover, if a large part of the chromosome is altered, it may affect cell viability. Several other assays have been developed to detect mutations by the selection of mutations in nonessential genes (37,38).

**Investigations of Cell Growth Regulation.** In normal cells, DNA damage is associated with interruption of cell cycle progression. Several check points control the cell cycle to avoid fixation of lesions in DNA or replication of damaged DNA. Some agents alter cell cycle control; this may result in deregulated expression of specific genes and impaired DNA repair, leading to chromosome damage.

## Mechanisms of Action of Fibers

### DNA Damage in Cell-free Systems

Deoxyguanosine hydroxylation was measured by the amount of 8-OHdG formed in calf thymus DNA. The experimental conditions differed among experiments but concentrations were generally several milligrams per milliliter for fibers and about 1 mg/ml DNA. Although it is difficult to

assay the amount of 8-OHdG/ $10^5$  dG formed in baseline conditions (fibers suspended in buffer alone), one can detect about ten 8-OHdG/ $10^5$  dG guanine hydroxylation by asbestos. All types of asbestos were tested (crocidolite, chrysotile, anthophyllite). The addition of  $H_2O_2$  enhanced the 8-OHdG yield, and the effect was potentiated in the presence of EDTA (39–41). Synthetic fibers [fiber glass, KTi whiskers, and other man-made mineral fiber(s) (MMMF)] also produced 8-OHdG, but experimental conditions were not uniform enough between studies to allow comparisons between fiber types (39,42).

Formation of 8-OHdG is likely due to the generation of active oxygen species, especially  $OH^\cdot$ , even in the absence of  $H_2O_2$ . As discussed,  $OH^\cdot$  generation may be due to the presence of iron at the fiber surface; crocidolite asbestos fibers are often used in these studies. Therefore, some authors have investigated the effect of DF and other iron chelators on 8-OHdG yield. Interestingly, no consistent results have been obtained. Although addition of DF reduced the percentage of hydroxylation (43), crocidolite depleted of iron produced more 8-OHdG than its normal counterpart (40). The state of iron reacting in these processes strongly influences the effects: reduced iron and mobilizable iron are believed to be important in the production of ROS (44–46). To be efficient, Fe(III) must be chelated in solution to permit its reduction (47).

DNA breakage has been observed following incubation of plasmid DNA (PM2 or  $\Phi$ X174) or calf thymus DNA with asbestos (Table 5). As observed for guanine hydroxylation, chelation of Fe(III) by DF

protects against DNA damage. Reexposure of DF-treated fibers to iron enhanced the yield of DNA breakage (50). Antioxidants prevented DNA breakage, suggesting that ROS are involved in this DNA damage.

### Cellular DNA Damage Produced by Fibers

A few studies have investigated DNA breakage following treatment of mammalian cells with asbestos. Some studies reported an absence of DNA damage (51,52) while others demonstrated DNA breaks (Table 6). These inconsistent results may be due to the different cell types and different methods of analysis. Using alkaline unwinding and ethidium bromide fluorescence, Kamp et al. (56) found that chrysotile and amosite produce DNA strand breaks in A549 and WI-26 cells. In these assays, production of  $OH^\cdot$ -like species after 30 min incubation correlated with further yield of DNA strand breaks. Iron plays a role in the formation of DNA damage but does not totally account for DNA breakage. DNA strand breaks were also detected in mouse CH310T1/2 cells treated with crocidolite (55) and human blood cells treated with crocidolite (43).

DNA breakage was demonstrated indirectly in rat embryo cells treated with crocidolite using a nick-translation method (57). In rat pleural mesothelial cells treated with crocidolite and chrysotile, activation of poly(ADP)ribose polymerase, an enzyme activated by DNA strand breaks, has been found (60). Moreover, an enhancement of DNA repair has been observed in the same type of cells, indicating that DNA damage has occurred following asbestos treatment. This effect may be cell-type specific, as

hepatocytes did not show repair of DNA following treatment with chrysotile (62).

More recently, some authors have investigated the formation of DNA adducts and guanine hydroxylation (8-OHdG) in cellular DNA (Table 6). DNA adducts of malondialdehyde were observed in DNA of *S. typhimurium* TA104 and rat lung fibroblasts, RFL-6, incubated with crocidolite and man-made vitreous fiber(s) (MMVF), rockwool, and MMVF21 (61). Guanine hydroxylation was found in DNA of human HL-60 and A549 cells. The yield of 8-OHdG was dependent on fiber concentration and duration of incubation when these parameters were tested. The magnitude of 8-OHdG was on the order of five 8-OHdG/ $10^5$  dG.

### Gene Mutation Assays

In most studies to date fibers were not directly mutagenic. Both chrysotile and amphiboles were tested on bacteria and some mammalian cells (Table 7). Glass fibers were not mutagenic to bacteria (65). Only one study reported weak mutagenicity of asbestos at the *HGPRT* locus in Syrian hamster lung cells (68). In other systems, the number of mutants was enhanced in comparison with untreated cells, but was not significantly different from background incidence. Mutations in bacteria were found using a different *S. typhimurium* strain (TA102) that is sensitive to oxidative damage. When treated with crocidolite but not with chrysotile, a significant enhancement of mutants was observed (41). However, using the same strain, tremolite fibers were not found to be mutagenic (67). TA102 contains A-T base pairs at the site of reversion, in contrast with other strains

**Table 5.** DNA breakage by asbestos in cell-free systems.

| System   | Assay   | Effect  | Reference           |
|--|---|---|---------------------|
| 0.8 $\mu$ g PM <sub>2</sub> DNA<br>50 $\mu$ g crocidolite $\pm$ tobacco smoke $\pm$ $H_2O_2$ | Ethidium bromide fluorescence: percentage of DNA developing strand breaks<br>Effect of antioxidants | Percentage of DNA strand breaks asbestos:<br>$H_2O_2$ = smoke (10%)<br>Potentiation with asbestos: asbestos + smoke (70%)<br>Inhibition by antioxidants | Jackson et al. (48) |
| 0.5 $\mu$ g $\Phi$ X174 DNA<br>1 mg/ml asbestos<br>$\pm$ 1 mM ascorbate                      | Electrophoresis: percentage of DNA with SSB<br>Effects of antioxidants (crocidolite)                | Without ascorbate: 0%<br>With ascorbate: crocidolite 19%, amosite 26%,<br>chrysotile 7%, tremolite 8%<br>Inhibition by antioxidants and DF              | Lund and Aust (47)  |
| 1 mg/ml calf thymus DNA<br>$\pm$ peroxides   | S1 nuclease activity: double strand breaks. Percentage of DNA hydrolyzed                            | Chrysotile and crocidolite hydrolyze DNA in the presence of peroxides   | Mahmood et al. (49) |
| 0.25 $\mu$ g $\Phi$ X174<br>20 $\mu$ g crocidolite<br>+ 1 mM ascorbate<br>+ EDTA             | DNA SSB (47)<br>DF-treated crocidolite exposed to several iron concentrations                       | Enhancement of DNA strand breaks<br>With 0, 3.0, and 5.5 nmol $Fe^{2+}$ /mg fibers:<br>21, 42, and 51% SSB, respectively                                | Hardy and Aust (50) |

SSB, single-strand breaks.

**Table 6.** DNA damage produced in asbestos-treated cells.

| System  | Assay   | Reference                  |
|---|---|----------------------------|
| HL-60 differentiated with phorbol myristate acetate   | 8-OHdG in DNA<br>Significant enhancement: $2.2 \pm 0.09$ and $2.9 \pm 0.11$ 8-OHdG/ $10^5$ dG for control and asbestos, respectively      | Takeuchi and Morimoto (53) |
| Crocidolite, 50 $\mu\text{g/ml}$  | 8-OHdG in DNA   | Chao et al. (54)           |
| A549 cells  | Significant enhancement: $1.0 \pm 0.4$ , $4.8 \pm 2.0$ , $10.4 \pm 2.0$ 8-OHdG/ $10^5$ dG at different fiber concentrations, respectively |                            |
| Crocidolite, 0, 1.5, and 3.0 $\mu\text{g/cm}^2$   | DNA breakage (FDA with ethidium bromide)  | Turver and Brown (55)      |
| C3H10T1/2   | Enhancement of DNA breakage   |                            |
| Crocidolite, 25–200 $\mu\text{g/cm}^2$  | DNA breakage (FDA with ethidium bromide)  | Kamp et al. (56)           |
| WI-26 <sup>a</sup> (SV-40 transformed): amosite (25, 250 $\mu\text{g/cm}^2$ ), chrysotile (250 $\mu\text{g/cm}^2$ ) | Enhancement of DNA breakage   |                            |
| A549 <sup>b</sup> : amosite, 25, 250 $\mu\text{g/cm}^2$   | Nick translation  | Libbus et al. (57)         |
| Rat embryo cells  | Significant enhancement with all types of particles   |                            |
| Crocidolite, 0.05–2 $\mu\text{g/cm}^2$  |   |                            |
| Riebeckite and glass fibers (code 100) 2 $\mu\text{g/cm}^2$   | DNA repair: dose-dependent enhancement  | Renier et al. (58)         |
| Rat pleural mesothelial cells   | Partial dependence on the production of ROS   | Dong et al. (59)           |
| Chrysotile, crocidolite (2, 4, 10 $\mu\text{g/cm}^2$ )  | Activation of poly(ADP)ribose polymerase  | Dong et al. (60)           |
| Rat pleural mesothelial cells   |   |                            |
| Chrysotile, crocidolite (2–20 $\mu\text{g/cm}^2$ )  | DNA breakage (FDA with ethidium bromide)  | Faux et al. (43)           |
| Human blood cells   | Dose-dependent reduction of double-strand DNA   |                            |
| Crocidolite, 10–500 $\mu\text{g/ml}$  | DNA adducts: enhancement of DNA adduct formation  | Howden and Faux (61)       |
| Rat lung fibroblasts (RFL-6), crocidolite, MMVF21(2, 5 $\mu\text{g/cm}^2$ )   | Depends on iron mobilization  |                            |
| <i>S. typhimurium</i> crocidolite (0.4, 0.8 mg), chrysotile (0.4 mg)  |   |                            |
| MMVF21, RCF1 (0.4 mg) (+ $\text{H}_2\text{O}_2$ )   | DNA breakage (alkaline elution)   | Fornace et al. (51)        |
| Human bronchial epithelial  | No effect   |                            |
| Amosite, crocidolite (25 $\mu\text{g/ml}$ )   | DNA breakage (alkaline elution); no DNA breakage  | Mossman et al. (52)        |
| Hamster tracheal epithelial cells, chrysotile (1–3 $\mu\text{g/ml}$ ), crocidolite (1–10 $\mu\text{g/ml}$ )         |   |                            |
| Rat hepatocytes   | DNA repair: no enhancement  | Denizeau et al. (62)       |
| Chrysotile, 10, 100 $\mu\text{g/ml}$  | DNA breakage (alkaline elution through hydroxyapatite columns)  | Kinnula et al. (63)        |
| TSV40 immortalized human mesothelial cells  | No breakage   |                            |
| Amosite, 0, 10, 25, 100 $\mu\text{g/cm}^2$  | DNA breakage (alkaline elution)   | Gabrielson et al. (64)     |
| Human mesothelial cells   | No breakage   |                            |
| Amosite, 1, 10, 100 $\mu\text{g/ml}$  |   |                            |

FDA, fluorometric analysis of DNA unwinding. <sup>a</sup>Human pulmonary epithelial. <sup>b</sup>Human bronchoalveolar carcinoma.

containing G-C base pairs at these sites (41). It may appear paradoxical that crocidolite fibers do not produce mutations in these strains since they produce guanine hydroxylation by redox reactions, as discussed. It is concluded that crocidolite produces DNA damage (8-OHdG) that is not detected in mutational assays. Chrysotile and several types of MMMF also produce 8-OHdG; the absence of mutagenicity observed in different test systems may be due to the inability of the target cells to convert this base change into mutations.

In recent investigations, chrysotile was mutagenic at the HLA locus of human lymphocytes (38).

### Chromosome Mutation Assays

Mutagenicity of asbestos has been studied with systems allowing the detection of gene deletions. Using gel electrophoresis, it was observed that asbestos produces

large deletions. In hamster human cell hybrid, A<sub>1</sub>, Hei et al. (37,72) found mutagenicity at the S1 locus associated with the loss of other chromosome markers. This mutation spectrum is different from that of spontaneous mutations and indicates that large deletions occur following asbestos treatment. In other systems, loss of heterozygosity (LOH) at the HLA-A locus was observed when human lymphocytes were treated with crocidolite and erionite, but not with chrysotile (38). Similarly, crocidolite produced LOH at the HLA-A locus in a mesothelioma cell line (73).

Fiber mutagenesis is mediated through oxygen-derived free radicals, as demonstrated by the protective effect of antioxidants (37,72). When using antioxidants acting either intracellularly or extracellularly, extracellular generation of active oxygen species accounts for most of the mutations detected (72).

### Chromosome Damage

The induction of chromosome damage by fibers has been recently reviewed by Jaurand (74). Asbestos fibers produce both structural and chromosome changes in most cell types. Table 8 summarizes the data. Chromosome breakage was detected in all rodent cell types treated with asbestos, including CHO, Syrian hamster embryo (SHE), Chinese hamster (CH) lung cells, and rat tracheal and mesothelial cells. Positive results were observed less frequently with human cells. With human bronchial epithelial cells, Kodama et al. (90) observed that chrysotile produced a low but significant enhancement of chromosome aberrations and micronuclei, and crocidolite produced a micronuclei increase limited to one incubation period. Chromosome damage was observed in human lymphocytes but not in human lymphoid cell lines following treatment with chrysotile (88,92). Olofsson and

**Table 7.** Gene and chromosome mutations produced by fibers.

| System                               | Fiber types                                    | Results   | Reference                  |
|--------------------------------------|--|---|----------------------------|
| <i>S. typhimurium</i> TA1538, TA1535 | Chrysotile, crocidolite, amosite, antophyllite | No mutagenicity   | Chamberlain and Tarmy (65) |
| <i>E. Coli</i> , several strains     | JM100, JM110                                   |   |                            |
| <i>E. Coli</i>                       | Tremolite (richterite)                         | With S9, mutation rate enhanced   | Cleveland (66)             |
| <i>S. typhimurium</i> TA102          | Chrysotile, crocidolite (UICC samples)         | Mutagenicity of crocidolite but not of chrysotile                             | Faux et al. (41)           |
| <i>S. typhimurium</i> TA102          | Tremolite                                      | No mutagenicity   | Athanasios et al. (67)     |
| CH lung cells                        | Chrysotile, crocidolite, amosite               | Weak mutagenicity at <i>HGPRT</i> locus                                       | Huang (68)                 |
| Adult rat liver cells                | Chrysotile, crocidolite, amosite               | No mutation at <i>HGPRT</i> locus   | Reiss et al. (69)          |
| SHE cells                            | Chrysotile, crocidolite                        | No mutation at <i>HGPRT</i> and Na <sup>+</sup> /K <sup>+</sup> ATPase loci   | Oshimura et al. (70)       |
| CHO cells                            | Crocidolite                                    | No mutation at <i>HGPRT</i> locus   | Kenne et al. (71)          |
| Human-hamster hybrid A <sub>1</sub>  | Chrysotile, crocidolite                        | Mutagenicity at S1 locus<br>No mutagenicity at <i>HGPRT</i> locus             | Hei et al. (37,72)         |
| Human lymphocytes                    | Chrysotile, crocidolite, erionite              | No mutagenicity (crocidolite, erionite)<br>Mutagenicity (chrysotile 50 µg/ml) | Both et al. (38)           |
| Mesothelioma cell line               | Crocidolite                                    | No mutagenicity<br>LOH (HLA-A locus)  | Both et al. (73)           |
| Human-hamster hybrid A <sub>1</sub>  | Chrysotile, crocidolite                        | Mutagenicity at S1 locus and other genes                                      | Hei et al. (37)            |
| Human lymphocytes                    | Chrysotile, crocidolite, erionite              | LOH (HLA-A locus) (crocidolite, erionite)                                     | Both et al. (38)           |
| Mesothelioma cell line               | Crocidolite                                    | LOH (HLA-A locus)   | Both et al. (73)           |

UICC, Union Internationale Contre le Cancer.

**Table 8.** Summary of assays conducted to investigate the effects of fibers on chromosomes.

| Chromosome changes  | Cells        | Fibers                    | Result <sup>a</sup> | Reference                    |
|---|--------------|---------------------------|---------------------|------------------------------|
| Structural damage, including micronuclei formation                        | Rodent cells | Crocidolite               | 7/8                 | (70,75-81)                   |
|   |              | Chrysotile                | 10/10               | (70,75,77,79-85)             |
|   |              | Other fibers <sup>b</sup> | 8/9                 | (67,70,75,77,78,80,81,86,87) |
|   | Human cells  | Crocidolite               | 1/5                 | (77,88-90)                   |
|   |              | Chrysotile                | 2/6                 | (77,88,89,90,92)             |
|   |              | Other fibers              | 2/5                 | (77,89,91,93)                |
| Numerical chromosome changes and polyploidy (binuclei formation included) | Rodent cells | Crocidolite               | 12/12               | (70,71,75-80,94-97)          |
|   |              | Chrysotile                | 8/8                 | (70,75,77,79,80,84,94,96)    |
|   |              | Other fibers <sup>c</sup> | 8/8                 | (67,70,75,77,78,80,94,96)    |
|   | Human cells  | Crocidolite               | 2/6                 | (77,88,90,96)                |
|   |              | Chrysotile                | 4/7                 | (77,88,90,96)                |
|   |              | Other fibers              | 3/5                 | (77,91,96)                   |

<sup>a</sup>Number of studies showing chromosome changes/number of studies (several studies using different cell types may have been published in one reference). <sup>b</sup>Amosite, tremolite, erionite, glass fibers. <sup>c</sup>Amosite, tremolite, erionite, glass fibers, RCF.

Mark (89) did not observe chromosome breakage in human mesothelial cells (HMC) treated with asbestos. Similarly, Lechner et al. (91) reported the occurrence of chromosome rearrangements (translocations, aneuploidy, exchanges) in the same cell type treated with amosite. However,

Pelin et al. (93) found structural damage to HMC chromosomes treated with amosite and reported that the response was dependent on the donor. The occurrence and extent of chromosome damage in human cells may depend on cell type. It must be noted that in contrast with rodent

cells, several studies using human cells were conducted with lymphocytes, a cell type unable to phagocytose fibers. It is remarkable that the results obtained with lymphocytes or lymphocyte-derived cell lines were negative unless other cell types (monocytes, polymorphonuclear leukocytes) were present in the incubation media (88,92). The results obtained with lymphocytes most likely reflect release of secondary mediators from phagocytic cells.

### Mitotic Abnormalities and Aneuploidy

Mitotic abnormalities have been reported following treatment of several types of mammalian cells with both natural and synthetic fibers. Mitotic disturbances were observed by microcinematography (98,99) or ultraviolet microscopy (81). Anaphase aberrations (bridges, lagging chromatin) were detected in SHE cells, V-79, and mesothelial cells (80,95,100,101). Pelin et al. (102) found that chrysotile and crocidolite produced a significant enhancement of the percentage of abnormal anaphases in HMC treated with chrysotile and crocidolite, but amosite produced only a slight increase. Similarly, Yegles et al. (103) observed that crocidolite and several samples of chrysotile produced a significant number of abnormal anaphases, but amosite and MMMF [refractory ceramic fibers (RCFs), MMVF] were inactive. The authors suggest that the absence of effect from some fiber types may be due to an insufficient number of relevant fibers. On the basis of the hypothesis of Stanton et al. (104) (length > 8 µm; diameter ≤ 0.25 µm), the number of long, thin fibers necessary to detect an effect should be  $2.5 \times 10^5$  fibers/cm<sup>2</sup>. These results emphasize the importance of fiber dimensions and are in agreement with other data on morphological observations of mitosis in cells that have phagocytized asbestos: chromosome movements were clearly impaired during chromosome segregation by the interaction between fibers and the keratin cage in epithelial cells (98,100). In contrast, short fibers that follow cell movements did not impair chromosome migration.

Aneuploidy and polyploidy have been observed frequently in asbestos-treated cells. Few data are available with MMMF. A summary of results was recently reported (74). Both chrysotile and crocidolite produce numerical chromosome changes in several types of rodent cells, including CHO, CH lung, SHE, rat tracheal and mesothelial cells (74), and

HMC (Table 8). No effect was found in human fibroblasts or a lymphoid cell line (77,88). In one study with human bronchial epithelial cells, binucleated cells were observed; however, no change in chromosome number in cells treated with chrysotile was found, and a small effect was observed with crocidolite (90). In SHE cells, aneuploidy is an important step in cell transformation (105). Trisomy of chromosome 11 following asbestos exposure of SHE cells has been described as a key event in asbestos-induced cell transformation. In a study of mitotic disturbances in SHE cells, Dopp et al. (81) observed kinetochore staining in the micronucleus formed after asbestos treatment, indicating that whole chromosomes have been lost. Numerical chromosome abnormalities have been observed in human (91) and rat mesothelial cells (84) treated with amosite and chrysotile, respectively.

### ***In Vivo* Studies of Genotoxicity**

Few *in vivo* studies have been conducted to date. Results are summarized in Table 9. The feeding of mice with asbestos did not result in chromosome abnormalities in germinal cells (106). It should be noted that in humans, oral exposure to asbestos does not seem to be associated with neoplasia. In a model where *Drosophila melanogaster* were fed with fibers, both types of asbestos and RCFs produced chromosomal changes (107,108).

### **Discussion**

Fibers have been considered nongenotoxic carcinogens because of early studies demonstrating a lack of mutagenicity in gene mutation assays. However, in the last few years, new tests better adapted to the study of fiber effects have been developed and evidence has accumulated that fibers may exert a genotoxic action. Most of the results have been obtained with asbestos; few data have been obtained with RCFs and MMVF.

### **Guanine Hydroxylation and DNA Breakage in DNA**

Several investigators have reported that all types of asbestos and MMVF produce OH<sup>•</sup> in buffered solutions (29,74). It has been recently demonstrated that the OH<sup>•</sup> formed is able to produce 8-OHdG in DNA both in cell-free systems using plasmid or calf thymus DNA or after incubation of mammalian cells with the fibers. The role of guanine hydroxylation in carcinogenesis is not clear; however, 8-OHdG can cause miscoding during DNA replication, resulting in mutations (109). Since early mutagenesis tests with bacteria have used strains rich in G-C at the mutation site, it appears paradoxical that 8-OHdG, when formed in these bacteria, did not produce mutagenesis. This failure likely is due to the nature of repair processes operating to correct these defects, as mutations can be induced by DNA damage because of error-prone DNA repair mechanisms.

The application of new methods to analyze DNA breaks has permitted detection of DNA strand breaks produced by fibers, both in cell-free systems and in mammalian cells. If ROS play a role in these mechanisms, they do not seem to be the only parameter of importance (Table 10). From the reported results, it seems that iron mobilized by chelators (e.g., EDTA and citrate) is responsible for the observed responses. DF, by chelating Fe(III), impairs the formation of Fe(II) from Fe(III) under reducing conditions. Following mobilization and reduction, Fe(II) can act in a Fenton reaction if the reacting compounds are present (46). Therefore, the ability of iron to produce ROS will depend on the amount of Fe(III) and Fe(II), as well as the presence of reducing and chelating agents. If this mechanism occurs *in vivo*, the situation is complicated by the fact that fibers may bind Fe(II), a natural ion in biological media. If iron is present at a fiber surface, it is logical to suggest that it may play a role

in the production of ROS. However, recent data have indicated that iron-free fibers also produce DNA breaks and guanine hydroxylation, which suggests that the redox reaction may be catalyzed by other components present in the incubation media.

Fibers releasing large amounts of iron, such as short amosite fibers or MMVF21, do not produce significant DNA damage in comparison with other fibers. In contrast, iron-free fibers such as RCF1 can produce DNA damage (110). Some authors conclude that there is a correlation between DNA damage and the amount of mobilized iron (50,61). However, in some experiments, the experimental conditions included the addition of H<sub>2</sub>O<sub>2</sub> (61), which enhances the sensitivity of the assay. The nature of the incubation medium certainly plays an important role in the generation of ROS, as emphasized by Chao et al. (54), who studied DNA damage in culture medium with different iron concentrations. Moreover, trace metals may interfere with redox reactions. In cell-free systems where DNA is mixed with the fibers, DNA adsorption at the fiber surface should also be considered (111), since it may influence the extent of DNA damage. The distance between the site of radical production and the site of DNA damage is of great importance, and DNA adsorption influences this distance. Alternatively, binding DNA at the fiber surface might mask or transform reactive sites.

ROS production by redox chemical reactions is not the only process by which ROS can be generated. Phagocytosis is a major phenomenon in which oxygen derivatives are produced; during this process, superoxide anion is generated following interaction with the plasma membrane (29). This is not limited to internalization of fibers and has been studied in bacteria as well as in phagocytic cells (112–114). Phagocytosis is associated with lipid peroxidation, as demonstrated by several authors in cell-free systems (31,32,115,116) and in mouse fibroblasts (55) and macrophages (117). Lipid peroxides are more stable molecules that may play a role in DNA damage.

The origin of the ROS generation is sometimes confusing. Several studies indicate that DNA damage is lowered by the addition of antioxidant enzymes superoxide dismutase and catalase. Others do not report such protection. With rat pleural mesothelial cells, Dong et al. (59) report that these enzymes exert only partial protection against DNA damage. Takeuchi and Morimoto (53) report that the formation

**Table 9.** *In vivo* tests used to study genotoxicity of fibers.

| Assay  | Result  | Reference                 |
|--|---|---------------------------|
| Mice fed chrysotile, 20 mg/kg/day, for 60 days—study of metaphases in germinal cells   | No effect   | Oshimura et al. (105)     |
| <i>Drosophila melanogaster</i> fed asbestos—study of offspring phenotypes that provides information on aneuploidy in oocytes | Aneuploidy with chrysotile, nonfibrous tremolite and amosite<br>Crocidolite: nonsignificant | Rita et al. (106)         |
| <i>Drosophila melanogaster</i> fed asbestos—fibers tested: RCF1, -2, -3, -4  | All samples produce aneuploidy in germinal cells  | Osgood and Sterling (107) |

**Table 10.** Role of oxidants and iron in DNA damage in cellular and cell-free systems.

| Method of investigation                                 | System   | Result   | Reference                  |
|---|--|--|----------------------------|
| Use of fibers treated with iron chelators               |  |  |                            |
| Desferrioxamine   | DNA SSB in $\Phi$ X174 RF (0.5 $\mu$ g) $\pm$ crocidolite 1 mg/ml  | Inhibition of DNA SSB by DF (in the presence of ascorbate)                     | Lund and Aust (47)         |
|   | 8-OHdG in calf thymus DNA (50 $\mu$ g/ml) + crocidolite (5 mg/ml)  | DF reduced production of 8-OHdG  | Faux et al. (41)           |
|   | Breakage of $\Phi$ X174 RF (0.24 $\mu$ g) $\pm$ asbestos and synthetic fibers (0.6, 0.9, $1.2 \times 10^6$ fibers) | Crocidolite, long amosite: DF reduces breakage RCF and MMVF: no effect from DF | Gilmour et al. (110)       |
|   | 8-OHdG in A549 treated with crocidolite (6 $\mu$ g/cm <sup>2</sup> )   | DF reduced the amount of 8-OHdG formed   | Chao et al. (54)           |
| Phytic acid   | DNA breakage in WI-26 and A549 cells $\pm$ amosite (25 $\mu$ g/cm <sup>2</sup> )                                   | No effect of phytic acid on DNA breakage produced by amosite                   | Kamp et al. (56)           |
| Addition of chelator (phytic acid) to incubation medium | DNA breakage in WI-26 A549 and alveolar epithelial cells $\pm$ amosite (25 $\mu$ g/cm <sup>2</sup> )               | Reduction of DNA breakage produced by amosite                                  | Kamp et al. (56)           |
| Addition of antioxidants to incubation medium           |  |  |                            |
| Mannitol  | Breakage of $\Phi$ X174 RF DNA $\pm$ crocidolite, long amosite (0.6, 0.9, $1.2 \times 10^6$ fibers)                | Reduction of DNA breakage  | Gilmour et al. (110)       |
| Enzymes (catalase, superoxide dismutase)                | 8-OHdG in HL60 cells treated with crocidolite (50 $\mu$ g/ml)  | No effect but inhibition of reactive oxygen species production                 | Takeuchi and Morimoto (53) |
|   | Nick translation in rat embryo fibroblasts   | Nucleoside incorporation   | Libbus et al. (57)         |
|   | DNA repair in rat pleural mesothelial cells $\pm$ chrysotile and crocidolite                                       | Partial reduction of DNA repair  | Dong et al. (59)           |
| Inhibition of phagocytosis by cytochalasine             | 8-OHdG in HL60 cells treated with crocidolite (50 $\mu$ g/ml)  | Reduction of 8-OHdG formation  | Takeuchi and Morimoto (53) |

of 8-OHdG in DNA of HL60 cells is more likely related to the internal production of ROS, as cytochalasin B blocked base hydroxylation, and antioxidant enzymes were inefficient. Oxygen derivatives are produced in the cell following particle internalization; species generated inside the cell may also damage DNA, either directly, or more probably, via the generation of stable secondary derivatives such as lipid peroxides (61). Therefore, DNA damage in a cell will depend on fiber chemistry, cell type, and environmental conditions. Iron can amplify the amount of ROS produced but is certainly not the only factor affecting these reactions. The phagocytic ability of the target cell, its antioxidant defenses, and membrane properties are of great importance. Moreover, fiber dimensions can play an important role. It has been demonstrated in some systems that ROS-associated cell damage is dependent on fiber dimensions. Nonfibrous particles were less active than fibers in the stimulation of  $O_2^{\cdot-}$  production by alveolar macrophages (118); ROS are implicated in the cytotoxicity of long but not short fibers to tracheal epithelial cells (119,120) and macrophages.

### Mechanisms of Chromosome Damage

Chromosome damage in terms of breakage, micronucleus formation, and chromosome mutations may be also related to the production of ROS by fiber-treated cells. In some studies where the effects of antioxidants have been investigated, antioxidants generally exerted a protective effect. In  $A_L$  hybrid cell systems, Suzuki and Hei (121) found that extracellular production of ROS is more likely involved, as intracellular antioxidants were unable to reduce the level of mutations. As discussed above, oxidants can produce secondary radicals or clastogenic factors that are more stable than active oxygen species and act at a site distant from their production. In a study where rat pleural mesothelial cells were treated with chrysotile, we found that the medium, after depletion of the remaining fibers, retained clastogenic activity. Control media from cells incubated without fibers or from fibers without cells did not produce similar effects (122). Clastogenic activity decreased when cells were incubated in the presence of antioxidant enzymes, suggesting that the formation of stable clastogenic factors was derived from the production of ROS.

Few data are available to determine whether chromosome missegregation and aneuploidy are related to the production of ROS. The formation of abnormal anaphases was not dependent on the presence of antioxidant enzymes (103). The physical presence of fibers in dividing cells seems a more likely mechanism by which fibers impair mitosis. In this context, it appears that phagocytosis is an important prerequisite for chromosomal damage. Following phagocytosis, asbestos fibers accumulate inside cells in the perinuclear region (15). When the cells undergo division, chromosome movement may be impaired by changes in cell shape triggered by the fibers. In these processes, fiber dimensions play a critical role. Samples enriched with long and thin fibers tend to produce abnormal anaphases (103) and aneuploidy-dependent cell transformation of SHE cells by unmilled glass fibers; milled fibers, though they phagocytosed at approximately the same rate, were not efficient (123).

### Conclusions

Evidence has now accumulated that fibers are genotoxic agents, based on their ability to produce DNA and chromosome damage.

ROS play a role in DNA and chromosome breakage and originate both from redox reactions occurring at the fiber surface and from cellular production of relevant molecules. The involvement of secondary, more stable molecules derived from ROS seems likely. The mechanisms of fiber genotoxicity depend on two variables: the fiber's chemical composition and cell environment, and the fiber structure, especially its fiber dimensions. Fiber dimensions influence genotoxicity potential in two aspects: first, by the modulation of ROS

production by cells, as short fibers produce fewer oxidants than long fibers of the same type; and second, by their influence on the production of chromosome abnormalities. In all these processes, phagocytosis is a critical event. The involvement of phagocytosis in genotoxicity sustains the postulated mechanisms of fiber toxicity and carcinogenicity, especially the hypotheses that frustrated phagocytosis of long fibers contributes to fiber toxicity.

If DNA damage is a marker of a genotoxic potential, then only persistent DNA

damage or changes resulting from error-prone repair will be of importance for cellular transformation. Repair of fiber-induced DNA damage is of great importance. Moreover, if genetically altered cells are stimulated to proliferate by external mitogenic factors, genetic instability will be enhanced; therefore, additional effects such as sustained cell proliferation may have an indirect genotoxic effect by increasing the pool of genetically altered cells and contributing to genetic instability (17).

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